NUCLEAR STAINING OF DERMATOPHYTES WITH THE FLUORESCENT DYE ACRIDINE ORANGE*

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The internal structure of hyphae of the dermatophyte group of fungi has been studied by the use of the electron microscope (1, 2). Nuclear staining of various other fungi has been reported by De Lamater (3) using basic fuchsin, and by Robinow (4) using the Giemsa stain. The fluorescent dye, acridine orange has been used to study the deep mycoses (10) as well as the nuclei in bacteria (5) and mammalian cells (6), and has been shown to stain the cellular ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) specifically. The purpose of the present study is to demonstrate the nuclei in hyphae and spores of three dermatophytes; *Microsporum canis*, *Trichophyton mentagrophytes*, *Trichophyton rubrum*, and to show specific staining of DNA in the nuclei by the use of control studies with the enzyme deoxyribonuclease (DNAase) which selectively degrades DNA.

MATERIAL AND METHODS

Three dermatophytes, *M. canis*, *T. mentagrophytes* and *T. rubrum* were grown on Sabouraud's media on glass slide cultures as follows. Rectangular pieces of Sabouraud's media were placed on sterile glass slides (one per slide), the edges inoculated with one of the above fungi, and a sterile coverslip applied. The preparations were placed on the shelves of a wire rack, in a tightly capped glass jar, the bottom of which contained water soaked filter paper to maintain a moistened atmosphere. After incubation at room temperature for 5 to 10 days, the coverslips were removed, the block of media discarded, and the mycelial growth on the glass slides and coverslips were fixed in absolute alcohol for 5 minutes, air dried and stored at room temperature until used.

Acridine Orange Staining

Glass slides or coverslips with a thin layer of mycelial growth on one side were subjected to the following procedure: immersion for 2 minutes in 0.05 M glycine-HCl buffer, pH 2.6; 4 minutes in 0.01% acridine orange (George T. Gurr Co. Ltd., London, England) in glycine-HCl buffer, rinsed in glycine-HCl buffer for 2 minutes, then mounted with a coverslip and a drop of 10% glycerine in glycine-HCl buffer. Acridine orange stain also was used in other buffers including sodium acetate-acetic acid buffer 0.2 M, pH 3.6 to 5.8 and M/15 phosphate buffer, pH 5.5 to 7.0. When staining was carried out in buffers of pH > 5.0 excessive red fluorescence occurred, this was removed by immersing the slide in a 0.1 M $CaCl_2$ solution for 1–3 minutes before washing finally in the appropriate buffer and mounting with a coverslip in the way previously described.

Fluorescence Microscopy

Slides were examined by use of a Leitz Aristophot ultra-violet light microscope with the following attachments: Dark field condenser, HBO 200 burner, excitor filter BG 12 and barrier filter OG 1, 2 mm. Photographs were taken with a Leitz 35 mm camera attachment using Ilford, HPS 400 black and white film.

Treatment with Enzymes DNAase and Ribonuclease (RNAase)

Mycelium on glass slides were incubated for 2–3 hours in a solution of 0.01% DNAase (Worthington Biochemical Corp., New Jersey, 1× crystallized) in veronal-acetate buffer M/40, pH 7.4 containing 0.01% gelatine and 0.003 M magnesium sulphate. After incubation at 37° C the sections were stained with acridine orange. Slides were also processed in a solution of 0.01% RNAase (Sigma Chemical Co., St. Louis, 5× crystallized) in the above veronal-acetate buffer by incubating at 37° C for 1½ hours before staining with acridine orange.

RESULTS

Three groups of dermatophytes, *M. canis*, *T. mentagrophytes* and *T. rubrum* were stained with the fluorescent dye, acridine orange and viewed through the ultra-violet light microscope. The hyphae appeared a red fluorescent color with yellow or green fluorescent nuclei in the hyphal segments. The nuclei appeared in an irregular arrangement both in number, size, shape and position in the hyphal segments (Fig. 1). Two to four discrete bright yellow fluorescent areas were

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This work was aided financially by the National Cancer Institute of Canada and the Foster Bequest Fund for Cancer Research.

Received for publication November 5, 1960.

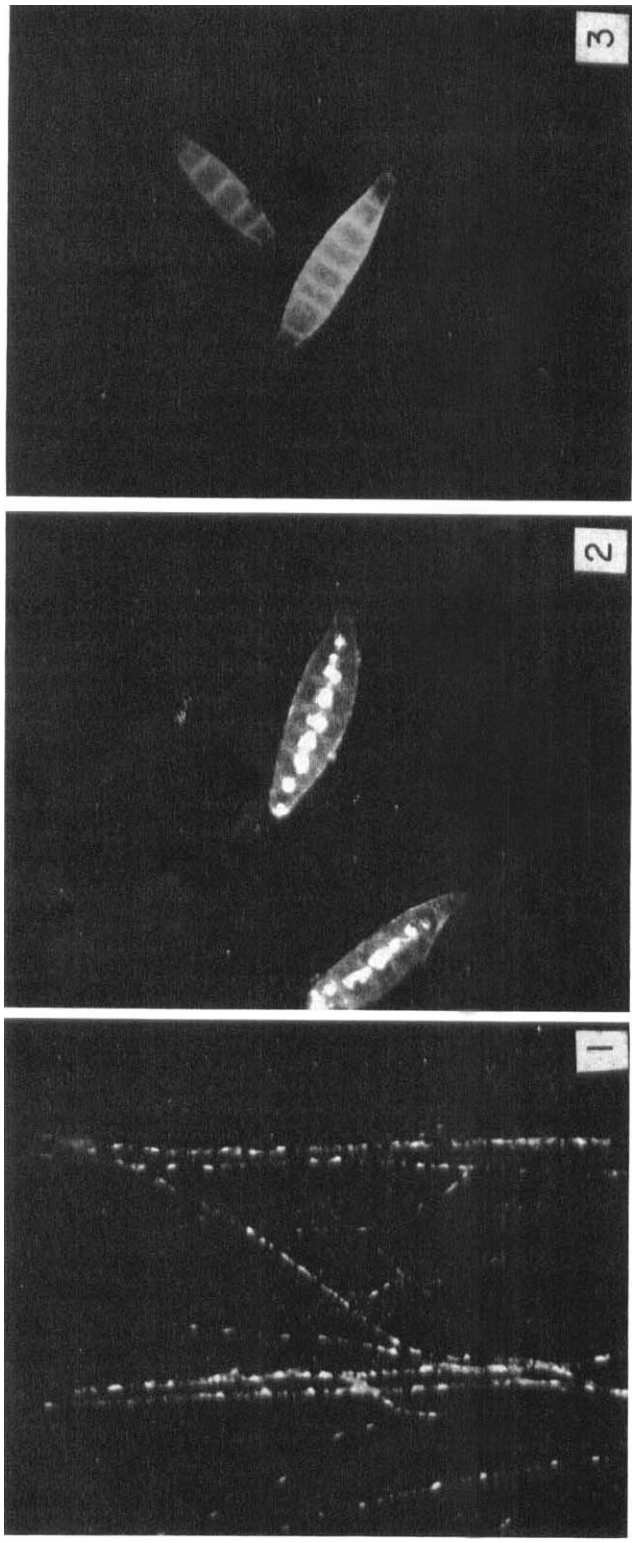

FIG. 1. Photograph of several hyphae from a culture of *Microsporum canis* stained with acridine orange. Viewed through the ultra-violet light microscope the hyphae appeared a red fluorescent color with yellow fluorescent nuclei in each segment. In the black and white photograph above, the nuclei appear white but the cellular outline of each hypha is not visible. Mag. × 456.

FIG. 2. Two macroconidia from a culture of *M. canis* stained with acridine orange showing a bright fluorescent nucleus in each segment of the macroconidia. Mag. × 760.

FIG. 3. Macroconidia from a culture of *M. canis* treated with DNAase and subsequently stained with acridine orange. The DNA content in the nuclei has been depolymerized by the enzyme and no longer takes up the yellow fluorescent dye. Mag. × 760.

seen in some hyphal segments but more frequently multiple diffuse, often granular, areas of less intense yellow or green fluorescence were seen, making it difficult to determine the exact number of nuclei present in each hypha. The area of nuclear fluorescence varied, most hypha contained yellow fluorescent areas of different size and shape. Nuclear fluorescence varied in intensity between nuclei of different hyphae and the nuclei of the same hypha. The site of nuclear fluorescence was not constant, yellow fluorescence was seen at the center, at the end or against the side of the cell wall. The nuclear staining of all three dermatophytes was similar; however *M. canis* (Fig. 1) showed areas of bright yellow fluorescence which often appeared larger and more discrete than the nuclei of the other two groups of dermatophytes. Intense yellow fluorescence was visible in the microconidia of *T. mentagrophytes* and *T. rubrum* as well as the macroconidia of *M. canis* (Fig. 2) which showed a discrete intense yellow fluorescent nucleus in each segment of these macroconidia.

Fungi (hyphae and conidia) treated with DNAase and stained with acridine orange showed no nuclear fluorescence (Fig. 3) but the red fluorescence of the body of the hyphal segment was visible. Treatment with RNAase followed by acridine stain showed bright yellow fluorescence of the nuclei with complete absence of any red fluorescence.

Fungi stained with acridine orange at pH 4.6 showed red fluorescence of the hypha and yellow or green fluorescent nuclei. With higher pH values, so much red fluorescence occurs that the whole hypha appears red. Differentiation in a solution of 0.1 M $CaCl_2$ is necessary to remove the excess red fluorescence in order to see the yellow nuclear fluorescence. At pH 2.6 little or no red fluorescence is seen but the nuclei stain intensely and this brighter yellow fluorescence stands out more clearly.

DISCUSSION

The technical difficulty involved in staining the nuclei in mycelial hyphae of the dermatophyte group of fungi is apparent from the paucity of reported nuclear staining technics. The fluorescent dye, acridine orange (2,8-bis-dimethylamine acridine) a fluorescent amino derivative of acridine has been shown to stain specifically, the nucleic acids, RNA and DNA in both plant (7) and animal cells (6, 8). In the present study, nuclei in the hyphae of the dermatophytes (*M. canis*, *T. mentagrophytes* and *T. rubrum*) stained with acridine orange and examined with the ultra-violet light microscope showed areas of yellow fluorescence specific for nuclear DNA. Evidence for the specific staining of the nucleic acid, DNA was shown by the result of treatment with DNAase after which the nuclei of hyphae so treated failed to show yellow fluorescence when subsequently stained with acridine orange.

The multinucleated character of the hyphal segment as well as their irregular size and shape has been shown from observations with the electron microscope (1). The absence of a nuclear membrane in fungi (1, 9) may explain the irregular distribution of DNA in the hyphal cell.

After various methods of fixation, attempts to stain the nuclei in hyphae with the Feulgen technic proved unsuccessful. De Lamater (3) reported unsatisfactory staining of hyphal nuclei with the Feulgen technic. Bakerspigel (9) reported Feulgen positive staining of the nuclei in hyphae and yeast cells of *Blastomyces dermatitidis*, however successful staining of the dermatophytes, with the Feulgen technic has yet to be reported.

The results of the present study show that the fluorescent dye, acridine orange is a simple method by which the nuclei in mycelial hyphae may be demonstrated. Hyphal segments of the dermatophytes (*M. canis*, *T. mentagrophytes* and *T. rubrum*) are multinucleated and the staining reaction of acridine orange indicated that these nuclei contain DNA.

SUMMARY

The fluorescent dye, acridine orange, was used to stain the dermatophytes, *M. canis*, *T. mentagrophytes* and *T. rubrum*. When viewed through the ultra-violet light microscope the hyphae appeared a red fluorescent color with green or yellow fluorescent nuclei. The multinucleated hyphal segments showed an irregular distribution of the individual nuclei which were uneven in size and shape. Hyphae treated with DNAase and subsequently stained with acridine orange showed no yellow fluorescent nuclei.

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